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Normal mucus formation requires cAMP-dependent HCO_3^- secretion and Ca^{2+}-mediated mucin exocytosis

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Key points

- HCO_3^- is required for gel-forming mucins to form normal mucus, but how?
- Two apparently separate signalling pathways are activated concurrently to bring mucus formation to completion: a Ca^{2+}-mediated pathway mainly directs goblet cell exocytosis, and an independent cAMP-mediated pathway stimulates HCO_3^- secretion to help discharge exocytosed mucus.
- cAMP-dependent HCO_3^- secretion fails, disrupting the normal formation and discharge of mucins in cystic fibrosis (CF) leading to pathologically viscous and tenacious mucus in affected organs.
- This work advances our understanding of the role of cAMP (CFTR)-dependent HCO_3^- secretion in forming normal mucus and underscores a new importance of addressing the defect in HCO_3^- secretion as a critical new therapeutic target in CF.

Abstract Evidence from the pathology in cystic fibrosis (CF) and recent results *in vitro* indicate that HCO_3^- is required for gel-forming mucins to form the mucus that protects epithelial surfaces. Mucus formation and release is a complex process that begins with an initial intracellular phase of synthesis, packaging and apical granule exocytosis that is followed by an extracellular phase of mucin swelling, transport and discharge into a lumen. Exactly where HCO_3^- becomes crucial in these processes is unknown, but we observed that in the presence of HCO_3^-, stimulating dissected segments of native mouse intestine with 5-hydroxytryptamine (5-HT) and prostaglandin E_2 (PGE_2) induced goblet cell exocytosis followed by normal mucin discharge in wild-type (WT) intestines. CF intestines that inherently lack cystic fibrosis transmembrane conductance regulator (CFTR)-dependent HCO_3^- secretion also demonstrated apparently normal goblet cell exocytosis, but in contrast, this was not followed by similar mucin discharge. Moreover, we found that even in the presence of HCO_3^-, when WT intestines were stimulated only with a Ca^{2+}-mediated agonist (carbachol), exocytosis was followed by poor discharge as with CF intestines. However, when the Ca^{2+}-mediated agonist was combined with a cAMP-mediated agonist (isoproterenol (isoprenaline) or vasoactive intestinal peptide) in the presence of HCO_3^- both normal exocytosis and normal discharge was observed. These results indicate that normal mucus formation requires concurrent activation of a Ca^{2+}-mediated exocytosis of mucin granules and an independent

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cAMP-mediated, CFTR-dependent, HCO_3^- secretion that appears to mainly enhance the extracellular phases of mucus excretion.

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Abbreviations CFTR, cystic fibrosis transmembrane conductance regulator; CF, cystic fibrosis; 5-HT, 5-hydroxytryptamine; PGE_2, prostaglandin E_2; CCH, carbachol; ISP, isoproterenol (isoprenaline); PAS, Periodic acid–Schiff; VIP, vasoactive intestinal peptide; WT, wild-type.

Introduction

The hallmark of cystic fibrosis (CF) is aggregated mucosal mucus in pancreas, intestines, liver, airways, reproductive organs and other mucus-producing exocrine glands (Oppenheimer & Esterly, 1975; Nicholson, 2002). In CF-affected organs, no uniquely characteristic changes in mucin composition have been identified (Mantle *et al.* 1990), and only the gel-forming mucins appear to be directly affected in CF pathology, indicating that a common defect caused by CFTR mutations leads to secondary pathogenic changes in these mucins (Quinton, 2001, 2008; Davis & Dickey, 2008; Garcia *et al.* 2009). Since the disease is caused by mutations in an ion transport gene (*CFTR*), the inevitable question remains as to how a defect in electrolyte transport results in abnormal mucus formation? Several prevalent hypotheses have been proposed to link the basic defect to the pathogenic, abnormally thick mucus in CF, but recently, defective CFTR-dependent HCO_3^- secretion was proposed to disrupt normal mucus gel formation (Quinton, 2001, 2008; Garcia *et al.* 2009; Chen *et al.* 2010; Muchekehu & Quinton, 2010; Gustafsson *et al.* 2012). Evidence of impaired HCO_3^- secretion has been reported in almost all CF-affected organs such as pancreas (Hadorn, 1968; Gaskin *et al.* 1982; Kopelman *et al.* 1988), intestine (Hogan *et al.* 1997; Seidler *et al.* 1997; Clarke & Harline, 1998), gallbladder (Cuthbert, 2001), reproductive tract (Chan *et al.* 2006) and airways (Smith & Welsh, 1992; Coakley *et al.* 2003). The extent to which HCO_3^- secretion is defective also appears to correlate with the severity of the CF phenotype (Choi *et al.* 2001; Reddy & Quinton, 2003).

We recently showed that normal mucus formation and release requires the presence of CFTR-dependent HCO_3^-, probably to sequester Ca^{2+} from condensed mucins as they expand and disperse (Garcia *et al.* 2009; Chen *et al.* 2010; Muchekehu & Quinton, 2010). That is, within goblet cell granules, Ca^{2+} and H^+ cations shield the densely packed fixed anionic sites of mucin molecules (Verdugo *et al.* 1987*a*,*b*). HCO_3^- probably destabilizes the compact mucins in granules by neutralizing H^+ and complexing Ca^{2+} cations allowing the electrostatic repulsion of unshielded anions to expand the macromolecular mucins into a mature mucus matrix. In CF, the defect in HCO_3^- transport and consequent lack of HCO_3^- (and CO_3^{2-}) appears to limit mucin expansion and impede its swelling, transportability and release into the lumen (Garcia *et al.* 2009; Chen *et al.* 2010; Muchekehu & Quinton, 2010; Gustafsson *et al.* 2012).

In the present study, we investigated the effects of cAMP and Ca^{2+}-mediated signalling pathways on mucus production to help determine the steps in mucus formation at which CFTR-dependent HCO_3^- transport becomes crucial. To help delineate how different signals affect mucus gel formation, we arbitrarily considered the intracellular processes up to the point that the mucin granule content emerges from the goblet cell as 'exocytosis', and the subsequent extracellular processes of mucin expansion, matrix maturation, and transport into a lumen as mucin 'discharge' (Fig. 1; Quinton, 2010).

Methods

Animals

Wild-type (WT) adult C57BL/6 mice (20–26 g) from our breeding colony were maintained on standard laboratory chow and allowed free access to food and water. *CFTR*^{tm1Kth} mice (CF mice) carrying the most common human *CFTR* mutation, ΔF508, on a C57BL/6 background were purchased (Case Western Reserve University), bred, and raised in our vivarium. CF mice were maintained on Peptamen Junior (Nestle Nutrition) *ad libitum* with access to Colyte and pellets. Mice were anaesthetized with ketamine (100 mg kg^{-1}) and xylazine (10 mg kg^{-1}) by subcutaneous injection for deep surgical anaesthesia. When the hind limb flexor withdrawal reflex ceased, 12 cm of ileum proximal to the caecum was quickly excised and the animal was immediately killed by cervical dislocation. The University of California San Diego Institutional Animal Care and Use Committee approved all procedures used in this study.

Solution composition

The following solutions were used in all experiments and dissection procedures, excluding the HCO_3^- secretion measurements. NaCl Ringer (HCO_3^- free) solutions contained in mM: 150 Na^+, 4.6 K^+, 1 Ca^{2+}, 1 Mg^{2+},

150 Cl^-, 2.5 PO_4^{x-} and 10 mM glucose. Glucose-free NaCl Ringer solution was the same except glucose was replaced by mannitol. For HCO_3^- Ringer solution, 25 mM Cl^- was substituted equimolar with HCO_3^-. All solutions were adjusted to, and maintained at, pH 7.4 by gassing to equilibrium with either 100% O_2 (glucose-free NaCl Ringer and NaCl Ringer solution) or 95% O_2/5% CO_2 gas (HCO_3^- Ringer solution) as appropriate during all protocols. The osmolality of all solutions was ∼285 mosmol kg^{-1}.

In our previous study, when 25 mM HCO_3^- was present only in the luminal perfusate, we did not detect an effect on stimulated mucus release (Garcia*et al.* 2009). Under those conditions, sufficient amounts of perfused luminal HCO_3^- apparently did not effectively reach the immediate sites of mucus secretion in the lumens of the crypts and possibly in inter-villar spaces of the intestinal wall. However, more recently the properties of the CF mouse ileal mucus appeared to normalize when secreted into a high concentration (∼100 mM) of luminal bicarbonate buffer (Gustafsson *et al.* 2012). Physiologically, HCO_3^- must be secreted from the contraluminal compartment by the intestinal epithelium to produce effects on mucins and mucus. Therefore, even though we do not know the concentration of HCO_3^- secreted during mucus formation, it was always present at 25 mM on the baso-lateral side of the intestine in the present study.

Mucus discharge

After excision, the ilea were placed in NaCl Ringer solution at room temperature and divided into a proximal and distal half (*ca* 6 cm each; Cook, 1965), which were assigned alternately as control and experimental segments. Nifedipine (1 μM) and indomethacin (10 μM) were included to prevent peristalsis and reduce endogenous prostaglandin release during tissue handling (Garcia *et al.* 2009). The lumen of each ileal segment was carefully flushed with cold, oxygenated, glucose-free NaCl Ringer solution to remove residual mucosal contents. The segments were mounted and perfused vertically in a custom-designed perfusion chamber (36 ± 1°C). The perfusates were collected at 5 min intervals for basal measurements while incubating in HCO_3^- or HCO_3^--free (NaCl) Ringer solutions during the initial 20 min of perfusion, and then after beginning the experimental protocols at 3 min intervals for an additional 20–30 min. To quantify the mucus content of the mucosal perfusates, we employed a wheat germ agglutinin–horseradish peroxidase (WGA-HRP) binding assay (lectin dot blot assay) on methanol-activated Immobilon-P film (Millipore, MA, USA) as described previously in detail (Garcia *et al.* 2009).

Goblet cell exocytosis

We estimated the effect of pharmacological agents with or without HCO_3^- on the loss of mucins from goblet cell stores by histological analysis. As described above, mouse ilea (about 12 cm) were excised and immediately divided into proximal and distal halves. For every experiment, each half was divided into three pieces (*ca* 2 cm each). One piece was freshly dissected and immediately snap frozen, and the other two pieces were treated according to experimental design, i.e. incubated with an agonist in either NaCl or $NaHCO_3$ Ringer solution. Previously, we found that most of the discharged mucus appeared in the perfusate within the 15 min of stimulation (Garcia *et al.* 2009). Therefore, in the present study, the tissue was stimulated with agonist in the bath for 15 min. After stimulation, the pieces of ileum were placed in OCT (frozen tissue matrix) and snap frozen between cold aluminum blocks (−80°C). Frozen tissue was sectioned at −20°C to 7 μm thickness and stained with Periodic acid–Schiff reaction (PAS) for carbohydrates. The mucin content of goblet cells, which stained intensely with PAS, was easily distinguished among the far more numerous lightly stained enterocytes. Stimulation diminished the amount of stained mucin content since the number as well as the total area of PAS-stained cells decreased. The ratio of the goblet cells and enterocytes along the villar surface is relatively fixed in similar locations (Kudweis *et al.* 1989). Therefore, we used the length of the villi to standardize and compare the number and area of the goblet cells. That is, we quantified the extent of goblet cell exocytosis along the villar surface by assessing both the decrease in the number of detectable (PAS+) goblet cells per length of villar surface (GCs mm^{-1}) as well as the decrease in total area of detectable (PAS+) goblet cells per length of villar surface (GA mm^{-1}) in each slide examined (Plaisancie *et al.* 1998). Both the length of villar surface and total area of goblet cells were measured with MetaMorph (Molecular Devices, Sunnyvale, CA, USA). Nine sections (slides) were chosen randomly for each specimen of intestine. For each condition (freshly dissected, stimulated in NaCl Ringer solution, and stimulated in $NaHCO_3$ Ringer solution), we sampled intestines from three mice. Therefore, 27 sections (slides) were analysed for each experimental condition. We compared changes in GCs mm^{-1} and GA mm^{-1} of freshly dissected intestine tissue with tissues stimulated in NaCl Ringer and stimulated in $NaHCO_3$ Ringer in WT and CF mice, respectively. We also compared tissues: (1) freshly dissected (unincubated), (2) incubated in NaCl Ringer without stimulation, and (3) incubated in HCO_3^- Ringer without stimulation, to confirm that there was no significant difference in GCs mm^{-1} or GA mm^{-1} between freshly dissected tissues and unstimulated tissues incubated in NaCl Ringer or in HCO_3^- Ringer solutions (i.e. for GCs mm^{-1}: freshly dissected *vs.* NaCl Ringer,

$P = 0.84$; freshly dissected *vs.* HCO_3^- Ringer, $P = 0.60$; for GA mm^{-1}: freshly dissected *vs.* NaCl Ringer, $P = 0.35$; freshly dissected *vs.* HCO_3^- Ringer, $P = 0.12$). Observers blinded to the source of the tissues independently performed image analyses for corroboration.

HCO_3^- secretion measurement (pH-stat titration of HCO_3^- flux)

Immediately after excision, intact ilea from WT and CF mice were immersed in, and rinsed with, ice-cold oxygenated NaCl Ringer solution; the mesentery was removed, and the ileum was slit open along the insertion of the mesentery and mounted between two lucite half-chambers of a Ussing chamber with an exposed area of 0.1 cm^2. Experiments were performed under continuous short-circuited conditions (voltage-current clamp, VCC 600; Physiological Instruments, San Diego, CA, USA). The serosal solution contained the following (in mM): 140 Na^+, 5.2 K^+, 1.2 Ca^{2+}, 1.2 Mg^{2+}, 120 Cl^-, 25 HCO_3^-, 2.4 $H_xPO_4^{x-}$, and 10 glucose. For the mucosal solution the phosphate buffer was deleted and 25 gluconate and 10 mannitol (in mM) replaced HCO_3^- and glucose, respectively. The unbuffered mucosal solution was bubbled with 100% O_2 and the serosal solution was bubbled with 95% O_2/5% CO_2 at pH = 7.4. Mucosal pH was maintained at 7.4 by a continuous pH-stat titration of an isosmotic solution containing 0.5 mM HCl (radiometer, Copenhagen). The rate of alkalization (HCO_3^- secretion) was calculated from the volume/time of the HCl-containing titrant solution required to maintain the mucosal pH at 7.4. Basal parameters were measured for 30 min, after which, selected agonists were added to the serosal compartment of the Ussing chamber. Measurements were recorded at 5 min intervals for the subsequent 20–30 min. The rates of mucosal HCO_3^- secretion were expressed as micromolar per centimetre squared per hour. Mean basal and peak values for consecutive 15 min periods were averaged, and simulated; HCO_3^- secretion was determined by subtracting mean basal values from mean peak values after adding an agonist. At the end of each experiment, 25 mM glucose was added to test the activity of the glucose–sodium co-transporter and thereby validate tissue viability. The integrity of specimens was shown by transepithelial hyperpolarization (Seidler *et al.* 1997).

Chemicals

Nifedipine was dissolved in dimethyl sulfoxide (DMSO), while indomethacin and prostaglandin E_2 (PGE_2) were dissolved in ethyl alcohol and added to serosal solutions as needed. All other agents were dissolved in Ringer solution directly. The final concentrations of DMSO and ethyl alcohol in solutions ranged between 1:1000 and 1:10,000 (vol./vol.). All chemicals were obtained from Sigma-Aldrich. All agonists were applied only to the basolateral side of tissues.

Statistics

Values are expressed as means ± standard error of the mean. Statistical comparisons were made using a two-tailed Student's *t* test for a single value between two groups: a two-tailed ANOVA test for a single value within three groups and a two-way ANOVA test for the mucus discharge results under different experimental conditions over a given period. A value of $P < 0.05$ was accepted as significantly different.

Results

Effect of HCO_3^- on stimulated mucin exocytosis in WT and CF ilea

In our earlier study, we did not detect significant mucus discharge from stimulated CF mouse ilea (Garcia *et al.* 2009), but the step in the process of mucus formation that

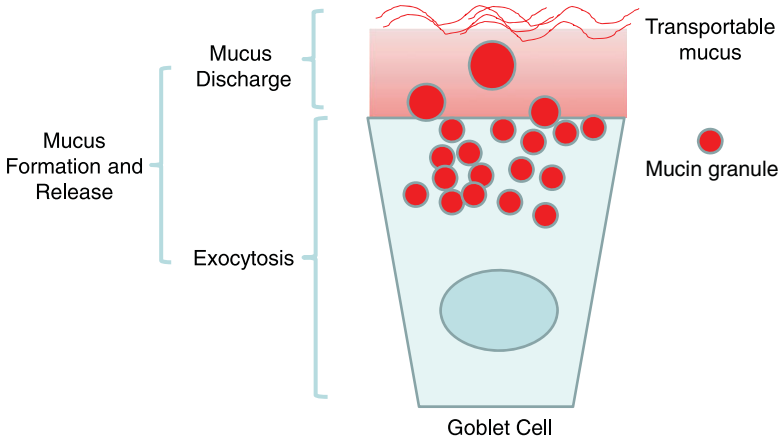

Figure 1. Exocytosis and discharge of mucus
In the present study, we arbitrarily consider the intracellular processes up to the point that the granule content begins to leave the goblet cell at the apical membrane as 'exocytosis', and the subsequent extracellular processes of mucin expansion, matrix formation, and transport into a lumen as 'discharge'.

fails in CF is not resolved. Therefore, we asked whether mucus formation/maturation in CF was impeded before or during mucin discharge. As a measure of induced exocytosis, we used 5-HT (10 μM) plus PGE_2 (1 μM) to obtain maximal mucus secretion as described previously (Garcia *et al.* 2009), and decreases in the cell number and area of PAS-stained goblet cells. In WT ilea incubated in NaCl Ringer solution and stimulated with PGE_2 plus 5-HT for 15 min, both the GCs mm^{-1} and the GA mm^{-1} were decreased about 20% compared to freshly dissected and frozen (unincubated) tissue ($n = 3$ mice, $P < 0.05$; Fig. 2*A* and *B*). Moreover, including HCO_3^- in the Ringer solution

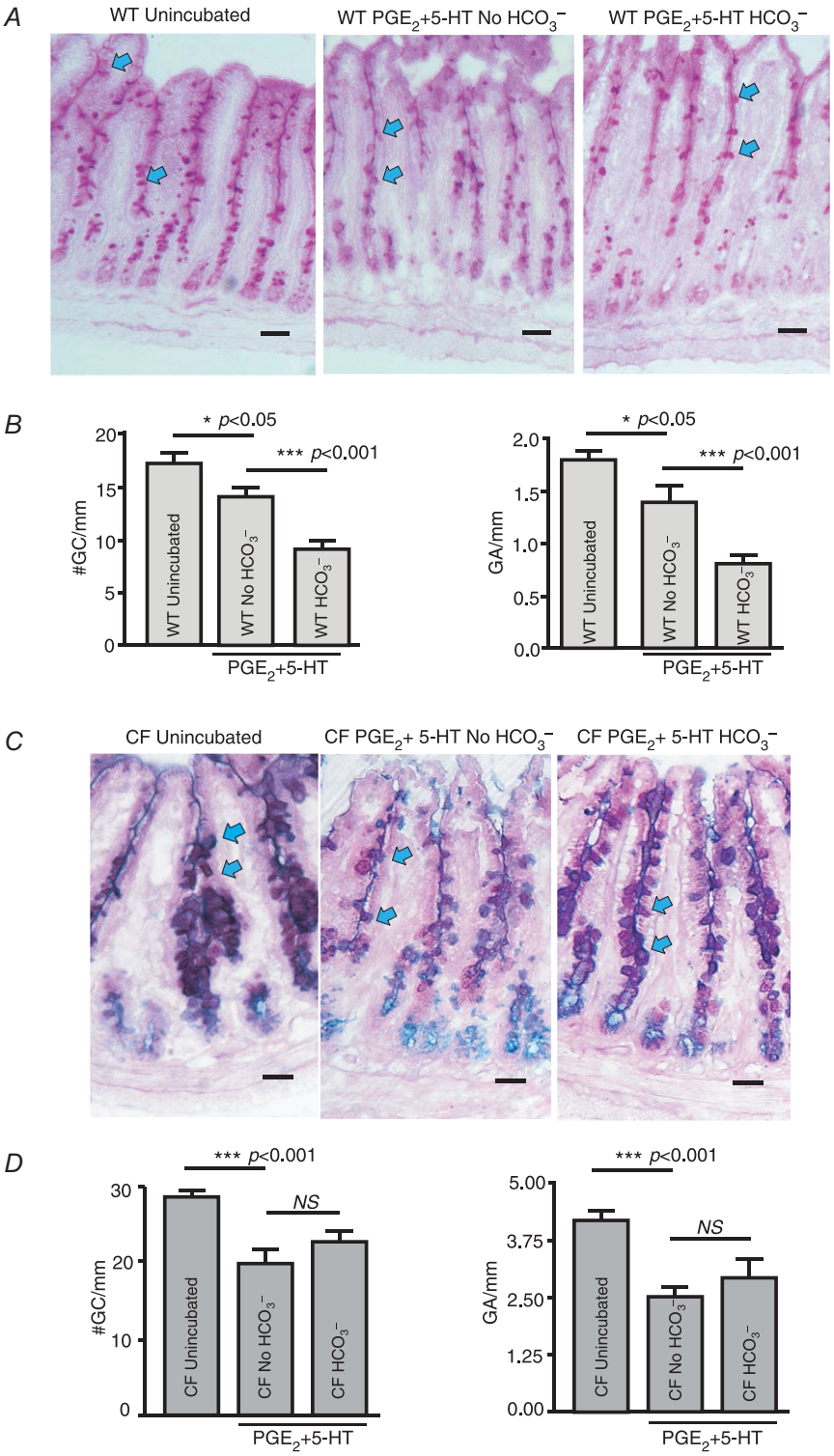

Figure 2. Effects of 5-HT plus PGE_2 on goblet cell exocytosis in WT and CF ilea with or without HCO_3^-
A, Periodic acid-Schiff (PAS)-stained frozen sections of unincubated WT ileum (left); PGE_2 (1 μM) + 5-HT (10 μM)-stimulated WT ileum in Ringer solution without HCO_3^- (middle); and PGE_2 + 5-HT-stimulated WT ileum in Ringer solution with HCO_3^- (right; scale bar = 50 μm). *B*, PGE_2 + 5-HT significantly decreased both GCs mm^{-1} (left) and GA mm^{-1} (right) compared with the unincubated WT ilea. HCO_3^- significantly further enhanced 5-HT + PGE_2-induced goblet cell exocytosis in WT ilea ($n = 3$ mice, means ± SEM.) *C*, Periodic acid-Schiff (PAS)-stained frozen sections of freshly unincubated CF ileum (left), PGE_2 (1 μM) + 5-HT (10 μM)-stimulated CF ileum in Ringer solution without HCO_3^- (middle), and PGE_2 + 5-HT-stimulated CF ileum in Ringer solution with HCO_3^- (right; scale bar = 50 μm). *D*, PGE_2 + 5-HT still significantly decreased both GCs mm^{-1} (left) and GA mm^{-1} (right) compared with unincubated CF ilea, whereas HCO_3^- in the bath solution did not enhance 5-HT- and PGE_2-induced CF goblet cell exocytosis ($n = 3$ mice, means ± SEM; NS, no significant difference; $P > 0.05$). Arrows indicate the PAS-stained goblet cells. GCs mm^{-1}, goblet cell number per length of villar surface; GA mm^{-1}, total goblet cell area per length of villar surface.

decreased both GCs mm^{-1} and GA mm^{-1} even further to about 50% of freshly dissected WT intestines ($n = 3$ mice, $P < 0.001$; Fig. 2*A* and *B*). These observations indicated that in the presence of HCO_3^-, stimulated WT goblet cell exocytosis is significantly enhanced.

Inherently, the GCs mm^{-1} and GA mm^{-1} were significantly larger in CF than in WT mouse ilea (Fig. 2*C* and *D*). As with WT, stimulation with 5-HT plus PGE_2 also significantly reduced the number and total area of goblet cells by about 1/3 in CF tissues incubated in NaCl Ringer solution, GCs mm^{-1} decreased by 28.6% ($n = 3$ mice, $P < 0.001$) and GA mm^{-1} decreased by 36% compared to freshly dissected CF ilea ($n = 3$ mice, $P < 0.001$; Fig. 2*C* and *D*). But, in contrast to WT, the presence of HCO_3^- did not enhance goblet cell exocytosis in CF ($n = 3$ mice, $P > 0.05$; Fig. 2*C* and *D*). These results indicate that CF goblet cells, like normal goblet cells, exocytosed in response to stimulation; however, exocytosed CF mucus does not competently discharge into the lumen since comparatively little mucus was detected in the mucosal perfusates of CF mouse intestines or cervices stimulated similarly (Garcia *et al.* 2009; Muchekehu & Quinton, 2010). To confirm that 5-HT and PGE_2-induced HCO_3^- secretion in WT mouse small intestine is CFTR dependent, we compared HCO_3^- secretion in CF ilea lacking CFTR with that of WT ilea. Similar to previous reports (Seidler *et al.* 1997; Clarke & Harline, 1998), 5-HT plus PGE_2 induced an increase in both short circuit current (I_{sc}) and HCO_3^- secretion in WT, but not in CF ileum (Fig. 3*A* and *B*). The basal HCO_3^- secretion in WT (3.22 ± 0.29, $n = 12$) was significantly higher than the basal HCO_3^- secretion in CF (0.99 ± 0.20, $n = 11$, $P < 0.0001$).

Effects of Ca^{2+}-mediated stimulation

Stimulation with PGE_2 plus 5-HT involves both cAMP and Ca^{2+}-mediated signalling pathways (Bonventre & Swidler, 1988; Cooke, 2000; Ning *et al.* 2004) with the activation of CFTR mainly associated with a cAMP pathway (Anderson *et al.* 1991; Quinton & Reddy, 1994). To better determine where CFTR-dependent HCO_3^- secretion contributes in the process of mucus formation and release, we attempted to dissect the effects of Ca^{2+}-mediated simulation from the effects of cAMP-mediated simulation on mucin exocytosis, mucus discharge, and HCO_3^- secretion.

To examine Ca^{2+}-mediated effects, we stimulated the ilea with carbachol (CCH, 100 μM), a cholinesterase-insensitive analogue of the well-established Ca^{2+}-mediated agonist acetylcholine (Fischer *et al.* 1992). Stimulation with CCH for 15 min significantly reduced detectable (PAS+) goblet cell number (GCs mm^{-1}) and total area of detectable (PAS+) goblet cells (GA mm^{-1}) of WT ileum in NaCl Ringer solution, similar to the effects of 5-HT plus PGE_2, i.e by 38% and 32%, respectively, compared to freshly isolated tissue ($n = 3$ mice, $P < 0.001$; Fig. 4*A* and *B*). However, CCH-stimulated exocytosis in tissues incubated in HCO_3^- Ringer solution did not increase compared to tissues incubated in NaCl Ringer solution ($n = 3$ mice; $P > 0.05$; Fig. 4*A* and *B*). In contrast to 5-HT and PGE_2, CCH induced only a modest mucus discharge in NaCl Ringer solution, which was similar to mucus discharged from ilea bathed in HCO_3^- Ringer solution ($n = 5$, $P > 0.5$; Fig. 4*C*). When we assayed for effects on HCO_3^- secretion, we found that CCH induced a sharp, transient increase in short circuit current without significantly stimulating HCO_3^- secretion (Fig. 4*D*). These results indicate that Ca^{2+}-mediated stimulation mainly induces goblet cell mucin exocytosis without stimulating significant HCO_3^- secretion.

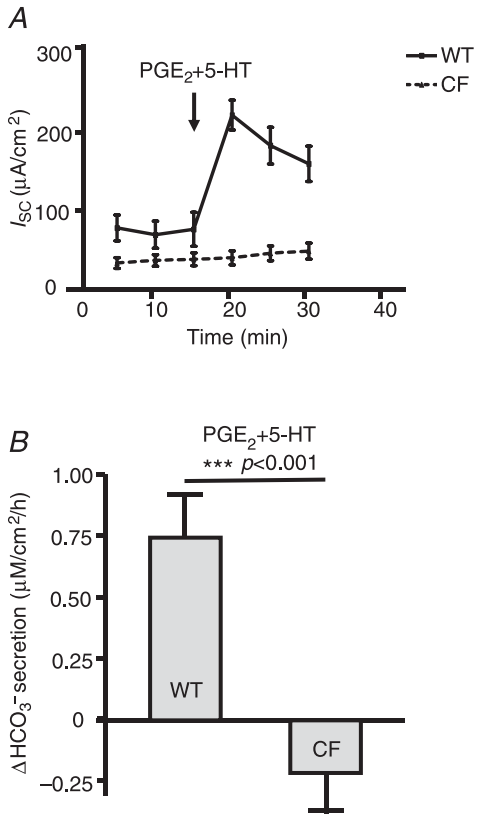

Figure 3. Effects of 5-HT plus PGE_2 on short circuit current and HCO_3^- secretion in WT and CF ilea
A, summary of stimulated short circuit current (I_{sc}) in WT and CF ilea. 5-HT (10 μM) plus PGE_2 (1 μM) induced an increase in short circuit current in WT ilea (continuous line; $n = 7$), but not in CF ilea (dashed line; $n = 7$). *B*, summary of stimulated HCO_3^- secretion in WT and CF ilea. 5-HT plus PGE_2 induced significant HCO_3^- secretion in WT ilea (0.72 ± 0.24, $n = 6$) but not in CF ilea (-0.22 ± 0.20, $n = 4$).

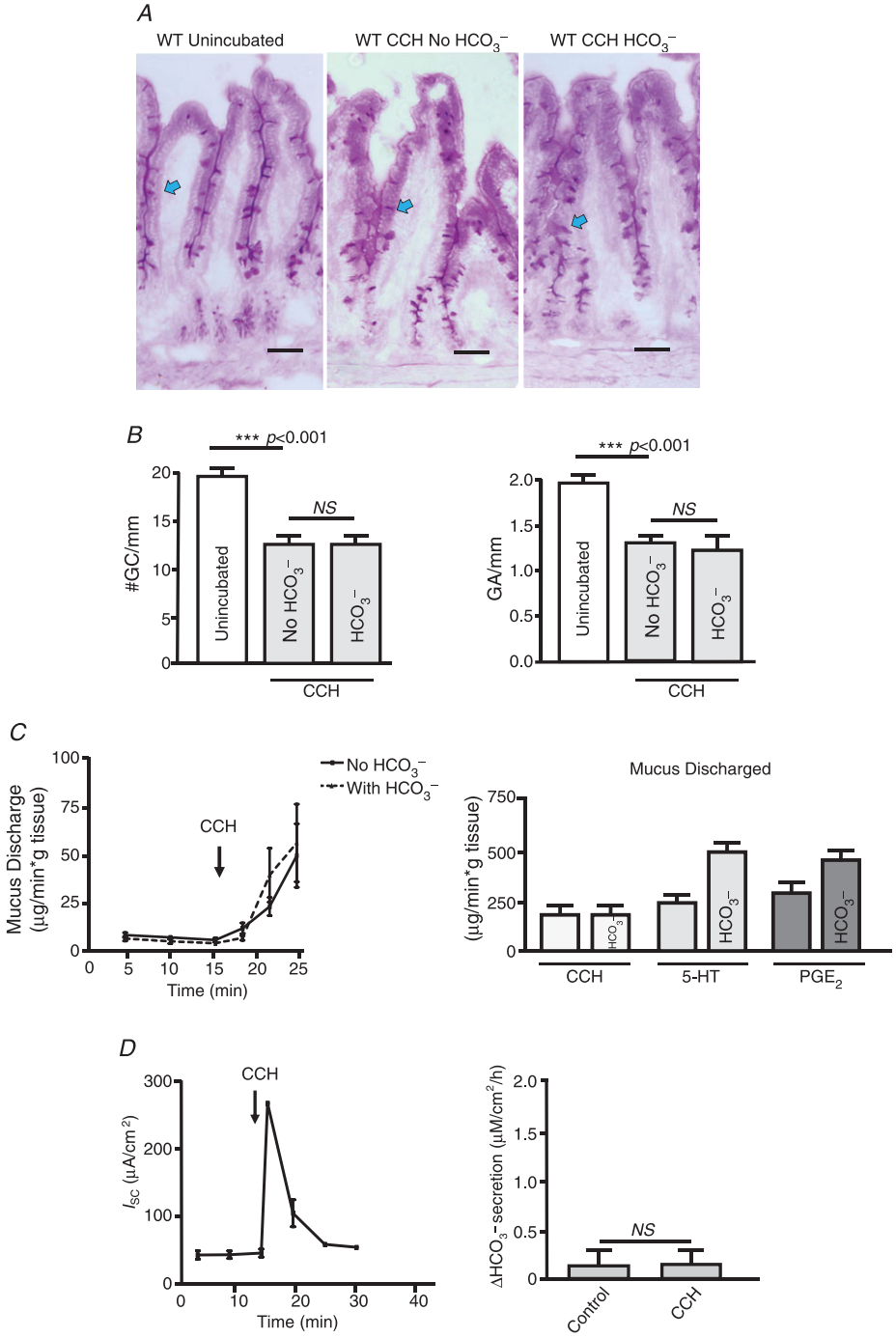

Figure 4. Effects of carbachol (CCH) on goblet cell exocytosis, mucus discharge and HCO_3^- secretion in WT mouse ilea

A, Periodic acid-Schiff (PAS)-stained frozen sections of unincubated WT ileum (left), CCH (100 μM)-stimulated WT ileum in Ringer solution without HCO_3^- (middle) and CCH-stimulated WT ileum in Ringer solution with HCO_3^- (right; scale bar = 50 μm); Arrows indicate the PAS-stained goblet cells. *B*, CCH decreased GCs mm^{-1} (left), and GA mm^{-1} (right) in WT ilea. HCO_3^- in the bath solution did not enhance CCH-induced exocytosis ($n = 3$ mice; mean ± SEM; NS, no significance, $P > 0.05$). *C*, comparison of CCH-stimulated mucus discharge with and without HCO_3^- in the bath solution (left). Comparison of CCH, 5-HT (10 μM) and PGE_2 (1 μM)-induced mucus discharge with and without HCO_3^- in the bath solution (right). Unlike the results of 5-HT or PGE_2, there is no significant increase in CCH-induced mucus discharged with HCO_3^- compared to no HCO_3^- in the bath. Samples were assayed by lectin binding dot blot ($n = 5$; $P = 0.32$). *D*, CCH induced a sharp and transient increase in short circuit current (left, $n = 7$); CCH did not increase HCO_3^- secretion compared to unstimulated control (right, $n = 5$; mean ± SEM; NS, not significant; $P = 0.99$).

Effects of cAMP-mediated stimulation

To determine the role of cAMP signalling on goblet cell exocytosis, mucus discharge and HCO_3^- secretion, we stimulated WT ilea with cAMP-mediated agonist isoproterenol (ISP), and confirmed with another cAMP-mediated agonist, vasoactive intestinal peptide (VIP). Neither ISP (10 μM) nor VIP (100 nM) alone induced significant goblet cell exocytosis, even in the presence of HCO_3^- (Fig. 5*A* and *B*), which was consistent with the results that ISP and VIP did not induce significant mucus discharge from ilea incubated in HCO_3^- Ringer solution either ($n = 4$, Fig. 5*C*). However, ISP and VIP stimulated marked increases in short circuit current and HCO_3^- secretion, which was not increased by adding carbachol (Fig. 5*D*). These results show that the effects of cAMP are mainly on HCO_3^- secretion and apparently occur independently of stimulating goblet cell mucin exocytosis.

cAMP-mediated HCO_3^- secretion potentiates discharge of Ca^{2+}-mediated exocytosed mucus

cAMP- and Ca^{2+}-mediated stimulation appear to have distinct roles in mucus formation and release, i.e. Ca^{2+} mainly involves activating goblet cell exocytosis, while cAMP mainly activates CFTR-dependent HCO_3^- secretion. We surmised that the effect of cAMP-activated CFTR-dependent HCO_3^- secretion must occur during the discharge process independently of the exocytosis process. We tested this notion by combining either ISP or VIP with CCH to stimulate mucus discharge in the presence and absence of HCO_3^-. We found that both VIP and ISP significantly augmented the CCH-simulated mucus discharge only when HCO_3^- was present in the bath solution ($n = 4$–5, Fig. 6*A* and *B*), but as shown above (Fig. 5*D*), CCH did not enhance ISP/VIP-stimulated HCO_3^- secretion. These observations indicate that Ca^{2+}-mediated mucin exocytosis is relatively independent of cAMP-mediated HCO_3^- secretion, but competent mucus discharge is highly dependent on concurrent cAMP-mediated HCO_3^- secretion.

Discussion

Although viscous, adherent mucus is pivotal in the development of cystic fibrosis pathology, how mutations in the *CFTR* gene lead to pathogenic mucus accumulation is only beginning to be understood. Over many years, several hypotheses have been proposed to explain the aetiology of abnormal mucus in CF, but we have observed that normal gel mucus release requires CFTR-dependent HCO_3^- secretion (Garcia *et al.* 2009; Muchekehu & Quinton, 2010). HCO_3^- may sequester Ca^{2+} from the mucin granule matrix to induce mucin gel dispersion and formation (Chen *et al.* 2010; Ambort *et al.* 2012). Gustafsson *et al.* (2012) very recently found that CF intestinal mucus appeared to form more normally and was less viscous when secreted into solutions of high HCO_3^- concentrations. Here, we have further examined whether HCO_3^- secretion and mucus exocytosis are independent processes under separate regulatory controls.

Mucin exocytosis *vs.* discharge in WT and CF

Our results showed that with HCO_3^- present, 5-HT plus PGE_2 stimulation induced goblet cell exocytosis, mucus discharge and HCO_3^- secretion in WT mouse small intestines, but that in CF intestines only exocytosis appeared to occur, suggesting that the intracellular mucin processes in CF goblet cells preceding exocytosis are probably not significantly impaired. Consequently, the abnormal mucus associated with the *CFTR* defect seems to occur mainly during mucus 'discharge' that depends critically on HCO_3^- secretion.

cAMP (CFTR)-dependent HCO_3^- secretion enhances mucus 'discharge'

As reported earlier, we used 5-HT and PGE_2 to induce robust mucus secretion (Garcia *et al.* 2009) and stimulate CFTR-dependent HCO_3^- secretion in mouse small intestine. 5-HT and PGE_2 are known to stimulate via both cAMP- and Ca^{2+}-dependent pathways (Bonventre & Swidler, 1988; Cooke, 2000; Ning *et al.* 2004) as observed in this study. Thus, we attempted to dissect the cAMP-dependent CFTR effects from the Ca^{2+}-mediated effects. Elevation of intracellular Ca^{2+} has been regarded as a key signal for exocytosis in most exocrine, endocrine and neuronal cells. Modulatory effects of cAMP pathways on Ca^{2+}-induced mucus formation and release have been reported for antral mucous cells and pancreatic ducts (Jung *et al.* 2010). Nakahari, *et al.* (2002) found that cAMP accumulation increased the number of primed granules and the sensitivity of granule fusion to Ca^{2+}, which potentiated Ca^{2+}-mediated exocytosis in antral mucous cells (Nakahari *et al.* 2002). Jung *et al.* (2010) also reported that Ca^{2+}-dependent exocytosis in cultured pancreatic duct epithelial cells (PDECs) was synergistically amplified by co-activation of cAMP-mediated signalling. However, increased cAMP did not modify the intensity or pattern of Ca^{2+} influx. Moreover, direct imaging of secretory granules did not reveal any effect of cAMP on the trafficking of these granules to the plasma membrane either, suggesting that the key influence(s) of cAMP may be on processes that take place at or very close to the plasma membrane (Jung *et al.* 2010). In the present study, when mucin exocytosis is stimulated, the

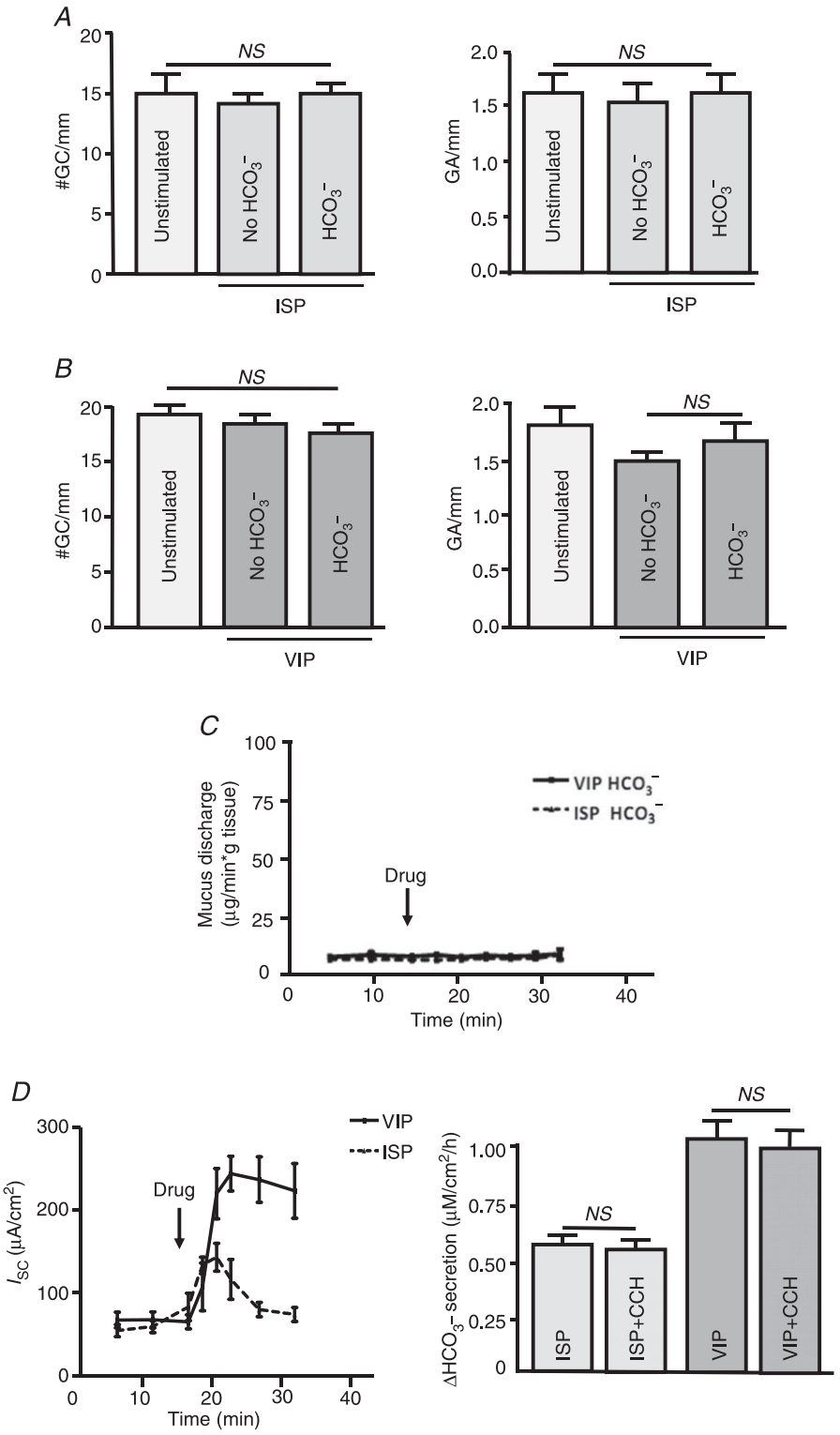

Figure 5. Effects of isoproterenol (ISP) and vasoactive intestinal peptide (VIP) on goblet cell exocytosis, mucus discharge, and HCO_3^- secretion in WT mouse ilea
Neither ISP (10 μM; *A*) nor VIP (100 nM; *B*) significantly reduced GCs mm^{-1} (left) or GA mm^{-1} (right) even in HCO_3^- Ringer solution ($n = 3$; mean ± SEM; NS, not significant, $P > 0.05$). *C*, neither ISP (10 μM) nor VIP (100 nM) induced mucus discharge even in HCO_3^- Ringer solution ($P = 0.13$, $n = 4$). *D*, left: both ISP (10 μM) and VIP (100 nM) significantly increased short circuit current ($n = 6$). Right: both ISP and VIP significantly increased HCO_3^- secretion ($n = 6$), but adding CCH (100 μM) did not enhance ISP- ($n = 4$) or VIP- ($n = 6$) stimulated HCO_3^- secretion (mean ± SEM; NS, not significant; ISP *vs*. ISP + CCH, $P = 0.98$; VIP *vs*. VIP + CCH, $P = 0.85$).

effect of cAMP on mucus discharge seems more likely to be due to concurrent, but separate CFTR-dependent HCO_3^- secretion based on the following findings: (1) cAMP-mediated stimulation significantly enhanced CCH-stimulated (Ca^{2+}-mediated) mucus discharge only in the presence of HCO_3^-, indicating that both cAMP signalling and HCO_3^- are essential for normal mucus 'discharge' (Fig. 6), (2) cAMP-mediated HCO_3^- secretion is CFTR dependent in the mouse ileum (Seidler *et al.* 1997; Clarke & Harline, 1998), (3) cAMP-mediated agonists by themselves did not induce mucus exocytosis or discharge (Fig. 5), and (4) in CF intestine, goblet cells exocytosed, but mucus discharge was compromised and incomplete even when stimulated in the presence of HCO_3^- (Fig. 2). These results strongly suggest that concurrent CFTR-dependent HCO_3^- secretion into the lumen is critical for exocytosed mucins to be 'discharged' normally.

Abnormally thick mucus in CF

Within intracellular mucin granules, high concentrations of Ca^{2+} and H^+ cations shield the high density of fixed anionic sites on the oligosaccharides that surround the protein cores of mucin monomers (Verdugo *et al.* 1987*a*,*b*; Perez-Vilar, 2007). During exocytosis, HCO_3^- is needed to sequester and remove high intragranular [Ca^{2+}] and [H^+] to allow normal mucin expansion and discharge (Garcia *et al.* 2009; Chen *et al.* 2010; Muchekehu & Quinton, 2010; Gustafsson *et al.* 2012). In CF, cAMP-activated CFTR-dependent HCO_3^- secretion is impaired because of dysfunctional or absent CFTR. However, as observed here, the Ca^{2+}-mediated exocytosis process appears to be intact in CF. Thus, exocytosed mucins apparently fail to expand normally when HCO_3^- is not present in the extracellular fluid into which they are exocytosed, leaving poorly expanded, aggregated mucus adherent to the mucosal surface (Garcia, 2009; Muchekehu & Quinton, 2010; Gustafsson *et al.* 2012). CF airway submucosal glands appear to retain mucus (Jayaraman *et al.* 2001; Joo *et al.* 2002), and the synergistic effects of cAMP on Ca^{2+}-mediated secretion are also absent in these glands (Choi *et al.* 2007), likewise indicating that cAMP-dependent HCO_3^- secretion is generally required to discharge Ca^{2+}-mediated exocytosed mucins. Concurrent stimulation of HCO_3^- secretion may well be an inherent property of exocrine organs that discharge gel-forming mucins.

Does CFTR-dependent HCO_3^- secretion also enhance goblet cell exocytosis?

Earlier we proposed that during mucin release the source of HCO_3^- was most likely to be the enterocytes surrounding the goblet cells in the epithelium (Garcia

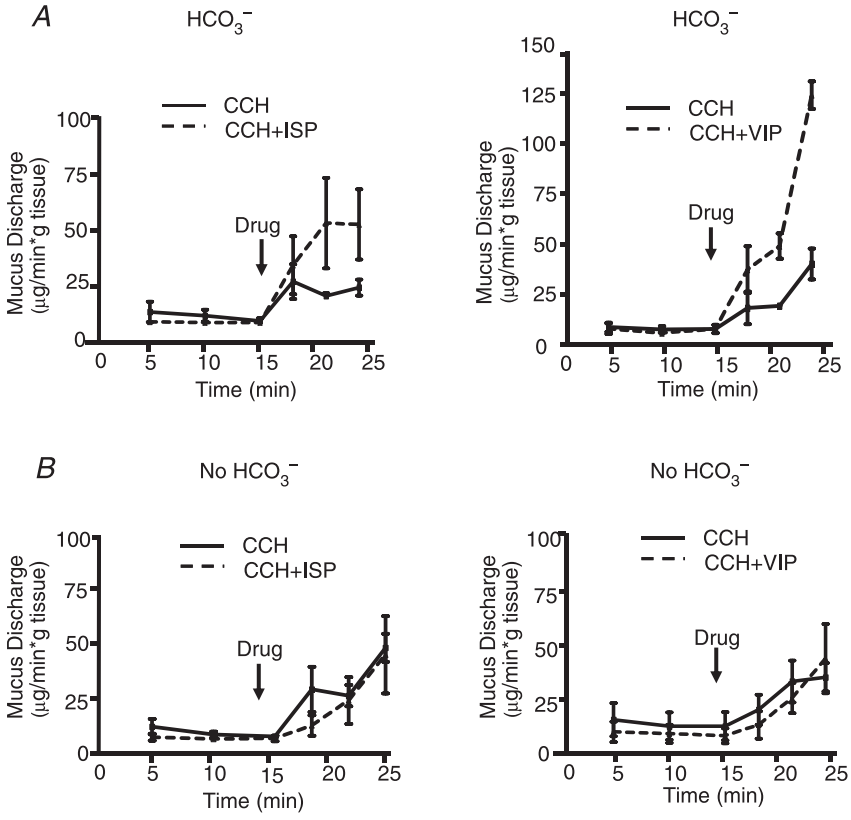

Figure 6 Effects of isoproterenol (ISP) and vasoactive intestinal peptide (VIP) on carbachol (CCH)-induced mucus discharge with or without HCO_3^- in WT mouse ilea
A, both ISP (10 μM) and VIP (100 nM) significantly enhanced CCH (100 μM)-induced mucus discharge when HCO_3^- was present in bath solutions. *B*, neither ISP nor VIP significantly increased CCH-induced mucus discharge when HCO_3^- was absent from bath solutions. Samples were assayed by lectin binding dot blot (means ± SEM; $n = 4$–5; CCH with HCO_3^- *vs.* CCH + ISP with HCO_3^-, $P = 0.03$; CCH with HCO_3^- *vs.* CCH + VIP with HCO_3^-, $P < 0.0001$; CCH No HCO_3^- *vs.* CCH + ISP No HCO_3^-, $P = 0.90$; CCH No HCO_3^- *vs.* CCH + VIP No HCO_3^-, $P = 0.91$).

et al. 2009). In this study, we show that mucin exocytosis from goblet cells is principally mediated by Ca^{2+}. However, in WT intestine stimulated with 5-HT plus PGE_2 (mediating both Ca^{2+} and cAMP signalling), somewhat more goblet cell exocytosis was observed with HCO_3^- present in the medium than without HCO_3^- (Fig. 2*B*). This enhancement was not seen in CF goblet cells (Fig. 2*D*), suggesting that goblet cell intracellular processes leading to exocytosis might partially depend on HCO_3^- and CFTR. Presently, the localization of CFTR in goblet cells remains controversial. Some investigators have reported CFTR immunolocalization within gastrointestinal goblet cells from humans (Kalin *et al.* 1999), pigs (Hayden & Carey, 1996), and mice (Hayden & Carey, 1996; Kuver *et al.* 2000), while others reported that CFTR is only expressed in neighbouring fluid-transporting epithelial cells (Jakab *et al.* 2011). Thus, aberrant mucus formation would be secondary to defective HCO_3^- secretion in enterocytes (Jakab *et al.* 2011). Yu *et al.* (2010) reported that a candidate Ca^{2+}-activated Cl^- channel protein in the basal membrane, bestrophin-2, may function in concert with a Cl^-/HCO_3^- exchanger localized in the goblet cell apical membrane for transcellular HCO_3^- transport, which might be regulated by external Cl^- supplied by CFTR in adjacent enterocytes (Yu *et al.* 2010). We cannot exclude the possibility that HCO_3^- may enter goblet cells and participate in initiating mucin granule exocytosis. Thus, the existence and role of CFTR in goblet cells requires further clarification.

Is CFTR the only source for HCO_3^- in the intestine?

Notably, in CF, mucus obstruction in the intestinal tract always occurs in the distal small intestine, not in the colon or duodenum, which suggests that different mechanisms or transport systems for HCO_3^- may exist in the distal small intestine *vs.* the colon or duodenum. Besides CFTR, Cl^-/HCO_3^- exchangers, short-chain fatty acid/HCO_3^- exchange, and basolateral HCO_3^- transporters also exist in the intestine (Nyberg *et al.* 1998; Talbot & Lytle, 2010; Bachmann & Seidler, 2011). In a recent study (Xiao *et al.* 2012), the expression of even small amounts of F508del-CFTR in the brush border membrane significantly enhanced the forskolin-induced HCO_3^- secretion response in murine duodenum and mid-colon. The secretion of HCO_3^- was dependent on luminal Cl^- in both intestinal segments, and therefore, probably due to Cl^-/HCO_3^- exchange. The results also indicate that Cl^-/HCO_3^- exchangers involve the HCO_3^- secretion in the duodenum and colon of CF mice, while the present study shows that in the mouse small intestine, CFTR may be the only, or at least the predominant, pathway for HCO_3^- transport associated with mucus formation in the ileum and that the level of spontaneous, exogenously unstimulated HCO_3^- secretion in CF ileal tissue is comparatively quite small. Taken together, these results may help explain the pathophysiology of the unique predisposition of distal small intestinal obstruction symptoms in CF mice and patients.

Conclusions

In summary, our results in mouse ileal intestine show that two distinct signalling pathways are required for normal mucus formation: a Ca^{2+}-mediated pathway appears mainly to induce goblet cell mucin exocytosis, while an independent cAMP-mediated pathway must concurrently stimulate HCO_3^- secretion, most likely in separate surrounding enterocytes. Physiologically, CFTR (cAMP)-dependent HCO_3^- secretion potentiates exocytosed mucin discharge in WT small intestine. However, in cystic fibrosis, cAMP-dependent HCO_3^- secretion, but not Ca^{2+}-mediated exocytosis, fails and disrupts the normal formation and discharge of mucus forming pathologically more viscous and tenacious mucus in affected organs.

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Additional information

Competing interests

The authors have no conflicts of interest to disclose.

Author contributions

The experiments were carried out at the University of California, San Diego. N. Y. designed and performed the experiments, analysed and interpreted the data and drafted and revised the manuscript. M.A.S.G. performed some of the Ussing-chamber and pH-stat experiments. P.M.Q. conceptually designed the experiments, critically interpreted the data and critically revised the manuscript. All authors approved the final version of the manuscript.

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